

# Localization of Distinct F<sub>2</sub>-Isoprostanes in Human Atherosclerotic Lesions

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## Abstract

F<sub>2</sub>-Isoprostanes are prostaglandin (PG) isomers formed in situ in cell membranes by peroxidation of arachidonic acid. 8-epi PGF<sub>2α</sub> and IPF<sub>2α</sub>-I are F<sub>2</sub>-isoprostanes produced in humans which circulate in plasma and are excreted in urine. Measurement of F<sub>2</sub>-isoprostanes may offer a sensitive, specific, and noninvasive method for measuring oxidant stress in clinical settings where reactive oxygen species are putatively involved. We determined whether isoprostanes were present in human atherosclerotic lesions, where lipid peroxidation is thought to occur in vivo. 8-epi PGF<sub>2α</sub> ranged from 1.310–3.450 pmol/μmol phospholipid in atherectomy specimens compared with 0.045–0.115 pmol/μmol phospholipid ( $P < 0.001$ ) in vascular tissue devoid of atherosclerosis. Corresponding values of IPF<sub>2α</sub>-I were 5.6–13.8 vs. 0.16–0.44 pmol/μmol phospholipid ( $P < 0.001$ ). Levels of the two isoprostanes in vascular tissue were highly correlated ( $r = 0.80$ ,  $P < 0.0001$ ).

Immunohistochemical studies confirmed that foam cells adjacent to the lipid necrotic core of the plaque were markedly positive for 8-epi PGF<sub>2α</sub>. These cells were also reactive with anti-CD68, an epitope specific for human monocyte/macrophages. 8-epi PGF<sub>2α</sub> immunoreactivity was also detected in cells positive for anti-α-smooth muscle actin antibody, which specifically recognizes vascular smooth muscle cells.

Our results indicate that 8-epi PGF<sub>2α</sub> and IPF<sub>2α</sub>-I, two distinct F<sub>2</sub>-isoprostanes and markers of oxidative stress in vivo, are present in human atherosclerotic plaque. Quantitation of these chemically stable products of lipid peroxidation in target tissues, as well as in biological fluids, may aid in the rational development of antioxidant drugs in humans. (*J. Clin. Invest.* 1997. 100:2028–2034.) Key words: atherosclerosis • oxidative stress • lipid peroxidation • isoprostanes

## Introduction

Considerable evidence implicates the oxidative modification of LDL in atherogenesis (1, 2). For example, various biological

activities thought relevant to the process are exhibited by oxidatively modified, compared with native LDL (3–8). Modified, but not native, LDL is avidly bound by macrophage scavenger receptors (9, 10) and recently, a receptor with particular affinity for oxidatively modified LDL has been described (11, 12). Additional to these findings, antibodies directed towards epitopes expressed in LDL oxidized in vitro have been shown to detect antigen in human atherosclerotic plaque (13) and to circulate in patients with advanced atherosclerosis. Despite these observations, there has been little direct evidence of a role for oxidant injury in atherogenesis. Indices of oxidant stress in vivo have been of limited value due to their chemical instability (14) or their nonspecificity (15, 16).

Isoprostanes are free radical-catalyzed products of arachidonic acid (17, 18). They are isomeric forms of the enzymatically generated prostaglandin species (19, 20). They are biochemically stable and, as such, have attraction as potential indices of oxidant stress in vivo. We have focused initially upon one of these compounds, 8-epi PGF<sub>2α</sub>, for which we have developed specific and sensitive methods of analysis, using gas chromatography/mass spectrometry (GC/MS).<sup>1</sup> We have observed increments in urinary 8-epi PGF<sub>2α</sub> in chronic cigarette smokers and during coronary reperfusion with thrombolytic drugs (21, 22), two syndromes putatively associated with oxidant stress (23, 24). We have also demonstrated that incubation of human monocytes with LDL results in marked, free radical-catalyzed formation of 8-epi PGF<sub>2α</sub> (25). More recently, we have established an assay for a second member of the F<sub>2</sub>-isoprostane family, IPF<sub>2α</sub>-I (26).

To explore further their potential as indices of oxidative stress, we decided to investigate whether 8-epi PGF<sub>2α</sub> and IPF<sub>2α</sub>-I were present in human atherosclerotic plaques. Consistent with the hypothesis that their production may reflect lipid peroxidation in the vessel wall, we have found a marked elevation of both 8-epi PGF<sub>2α</sub> and IPF<sub>2α</sub>-I content in atherosclerotic plaque obtained at endarterectomy. 8-epi PGF<sub>2α</sub> was also detected immunohistochemically in lipid-rich atherosclerotic lesions, predominantly associated with macrophages and smooth muscle cells. These observations support the hypothesis that measurement of these isoprostanes may provide a quantitative index of oxidant stress in human atherosclerotic disease in vivo.

## Methods

**Sample acquisition and tissue processing.** Human atherosclerotic plaques were obtained from 12 patients undergoing carotid endarterectomy. Informed consent was obtained from all patients before surgery. All procedures were approved by the local human ethics committee.

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1. Abbreviations used in this paper: GC/MS, gas chromatography/mass spectrometry; IPF<sub>2α</sub>-I, isoprostane F<sub>2α</sub> class I.

Table I. Clinical Characteristics of the Study Populations

	Patients (12)	Controls (8)
Age	T 54-76 (68) yr	43-56 (52) yr
Sex	T Male (12/12)	Male (8/8)
Risk factors		
Smoking	5/12	No
Hypertension	6/12	No
Diabetes	5/12	No
Hypercholesterolemia	8/12	No
Carotid stenosis	60-90%	No
Clinical history	Transient ischemic attacks in the last 6-12 mo (12/12)	Dilated cardiomyopathy (8/8)

Healthy tissue is not removed during endarterectomy, so comparison of diseased tissue with apparently uninvolved carotid vasculature obtained at endarterectomy is not possible. Vascular tissue, apparently devoid of plaque, was obtained from patients undergoing heart transplantation for dilated cardiomyopathy (aged 43-56; all males,  $n = 8$ ). These were materials obtained incidentally during the course of the transplant, but were only studied in subjects who gave informed consent. The area of the control vessels analyzed always included both the intima and the media. The patients undergoing endarterectomy were older (aged 54-76; all males) than the controls. Their clinical characteristics are summarized in Table I. We also studied carotid artery specimens obtained at autopsy performed within 4-8 h of death (three males who died in traffic accidents, aged 52-67; no disease or medication was noted in their medical records). Specimens were divided into normal intima and atherosclerotic lesions, based on macroscopic inspection. Both types of material were collected from each subject. The samples of normal intima (plus inner media) were obtained from regions which were free of all visible lesions. Although fatty streaks were not visible, there remains the possibility that they contained some isolated foam cells.

Freshly excised tissue was immediately placed into 0.15 NaCl (pH 7.0) containing 1 mM EDTA, 0.1 mM butylated hydroxytoluene, 100 U penicillin, 100  $\mu$ g streptomycin, and rinsed three times to remove any loosely adherent blood components. The presence of butylated hydroxytoluene and EDTA is important to prevent *ex vivo* formation of isoprostanes during the procedure. 10  $\mu$ g of [ $^3$ H]<sub>6</sub>arachidonic acid was also added to the samples, at the time of the acquisition, to detect any artifactual formation of 8-epi PGF<sub>2 $\alpha$</sub>  or IPF<sub>2 $\alpha$</sub> -I that might occur during sample extraction and processing. After homogenization with a blade homogenizer (four cycles for 30 s), total lipids were extracted with 20 ml ice-cold Folch solution, chloroform/methanol (2:1, vol/vol) (27). The solution was then vortexed and centrifuged at 800 g for 10 min at 4°C. An aliquot was taken to measure the total phospholipid content in the tissue, as previously described (28). The organic phase, containing the extracted lipids, was dried under nitrogen, then 5 ml of aqueous KOH (15%) was added and the mixture incubated at 45°C for 1 h to effect hydrolysis and release of total 8-epi PGF<sub>2 $\alpha$</sub>  and IPF<sub>2 $\alpha$</sub> -I. Total levels of each isoprostane were measured as described below.

**Biochemical analysis.** 8-epi PGF<sub>2 $\alpha$</sub>  and IPF<sub>2 $\alpha$</sub> -I were assayed by GC/MS, as previously described (21, 26). The intraassay and the interassay variability is  $\pm 3\%$  and  $\pm 4\%$  for 8-epi PGF<sub>2 $\alpha$</sub>  and  $\pm 4\%$  and  $\pm 5\%$  for IPF<sub>2 $\alpha$</sub> -I, respectively. In brief, a known amount of the internal standards [ $^{18}$ O]<sub>2</sub>8-epi PGF<sub>2 $\alpha$</sub>  and [ $^3$ H]<sub>6</sub>IPF<sub>2 $\alpha$</sub> -I was added to each sample. After solid phase extraction, the samples were derivatized and purified by two thin layer chromatography steps. Each sample was analyzed on a Fisons MD-800 (Fisons Instruments, Milan, Italy) GC/MS. Quantification was performed using peak area ratios.

**Immunohistochemistry.** After the surgical procedure, samples were immediately frozen in isopentane and cooled in liquid nitrogen. Serial fresh cryostatic sections were fixed in 10% phosphate-buffered formaldehyde for 5 min. Subsequently, they were washed in Tris-buffered saline (TBS) solution and incubated with normal goat serum (Ylem, Avezano, Italy), to block any nonspecific binding before staining. Sections were incubated with a specific rabbit polyclonal antibody anti-8-epi PGF<sub>2 $\alpha$</sub>  (1:400) for 1 h. The specificity and sensitivity of this antibody has been described previously (29). Biotinylated goat anti-rabbit IgG (Ylem) was used as a secondary antibody at 1:100 dilution. After washing twice with TBS, sections were treated with streptavidin-peroxidase complex for 30 min, then washed with TBS and the 3,3'-diaminobenzidine tetrahydrochloride (Sigma Chemical Co., St. Louis, MO) was used as final chromogen. To exclude the possibility of cross-reactions, an aliquot of the anti-8-epi PGF<sub>2 $\alpha$</sub>  antibody was added to an equal volume of a solution of 8-epi PGF<sub>2 $\alpha$</sub> . The sections incubated with this solution were completely negative. Two serial sections, adjacent to those used for reaction with the anti-8-epi PGF<sub>2 $\alpha$</sub>  antibody, were incubated with the monoclonal primary antibody anti-CD68 (anti-human monocyte/macrophages) (Dakopatts, Glostrup, Denmark) (30) to characterize the phenotype of the positive cells and the anti- $\alpha$ -smooth actin antibody (Dakopatts), which recognizes vascular smooth muscle cells (31). These antibodies were used according to the manufacturer's instructions, using the peroxidase antiperoxidase and the avidin-biotin-peroxidase complex methods with diaminobenzidine as a final chromogen. The hematoxylin staining of the nuclei was performed in all sections. Two negative controls were performed, either omitting the primary antibody or using a preimmune rabbit serum.

## Results

**8-epi PGF<sub>2 $\alpha$</sub>  in human atherosclerotic plaque.** All of the atherosclerotic plaque samples analyzed contained higher levels of 8-epi PGF<sub>2 $\alpha$</sub>  and IPF<sub>2 $\alpha$</sub> -I than the healthy vessels. Preoperatively, all of the specimens had the appearance of fibrocalcific plaques on carotid ultrasound. The levels of 8-epi PGF<sub>2 $\alpha$</sub>  in atherosclerotic plaque obtained at endarterectomy ranged from 1.310 to 3.450 pmol/ $\mu$ mol phospholipid, with a median of 2.25 pmol/ $\mu$ mol phospholipid. The corresponding values in the normal vessels ranged from 0.045 to 0.115 pmol/ $\mu$ mol phospholipid with a median of 0.090 pmol/ $\mu$ mol phospholipid ( $P < 0.001$ ) (Fig. 1). Levels of 8-epi PGF<sub>2 $\alpha$</sub>  in specimens obtained at

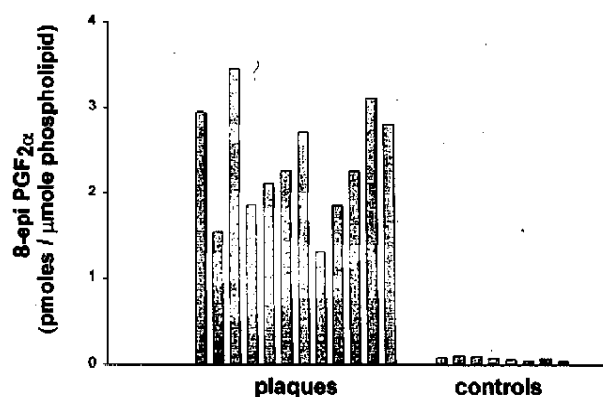


Figure 1. Levels of 8-epi PGF<sub>2 $\alpha$</sub>  in human carotid atherosclerotic plaque ( $n = 12$ ) and apparently healthy vascular tissue. Tissues were processed as described in Methods and assayed by GC/MS (four aortae, four pulmonary arteries).

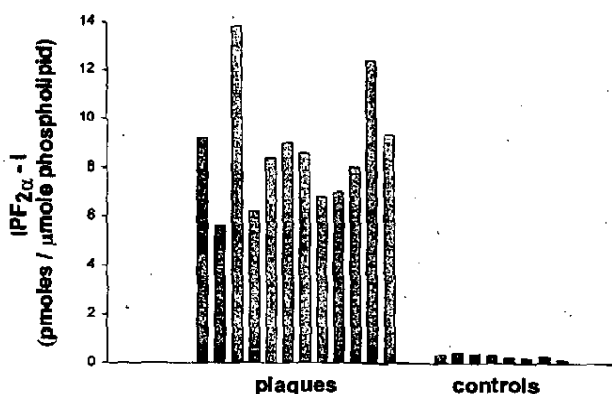


Figure 2. Levels of IPF<sub>2α</sub>-I in human carotid atherosclerotic plaque ( $n = 12$ ) and apparently healthy vascular tissue. Tissues were processed as described in Methods and assayed by GC/MS (four aortae, four pulmonary arteries).

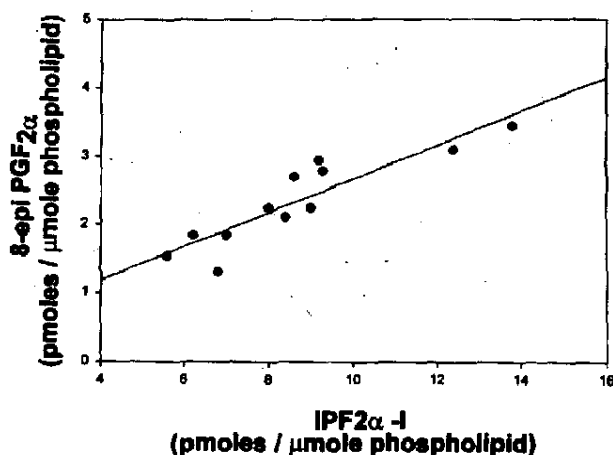


Figure 3. Correlation between levels of 8-epi PGF<sub>2α</sub> and IPF<sub>2α</sub>-I in the same atherectomy specimens ( $r = 0.80$ ,  $P < 0.0001$ ).

autopsy ranged from 1.85 to 3.60 in the atherosclerotic segments and from 0.06 to 0.150 pmol/μmol phospholipid in the corresponding normal intimal segments.

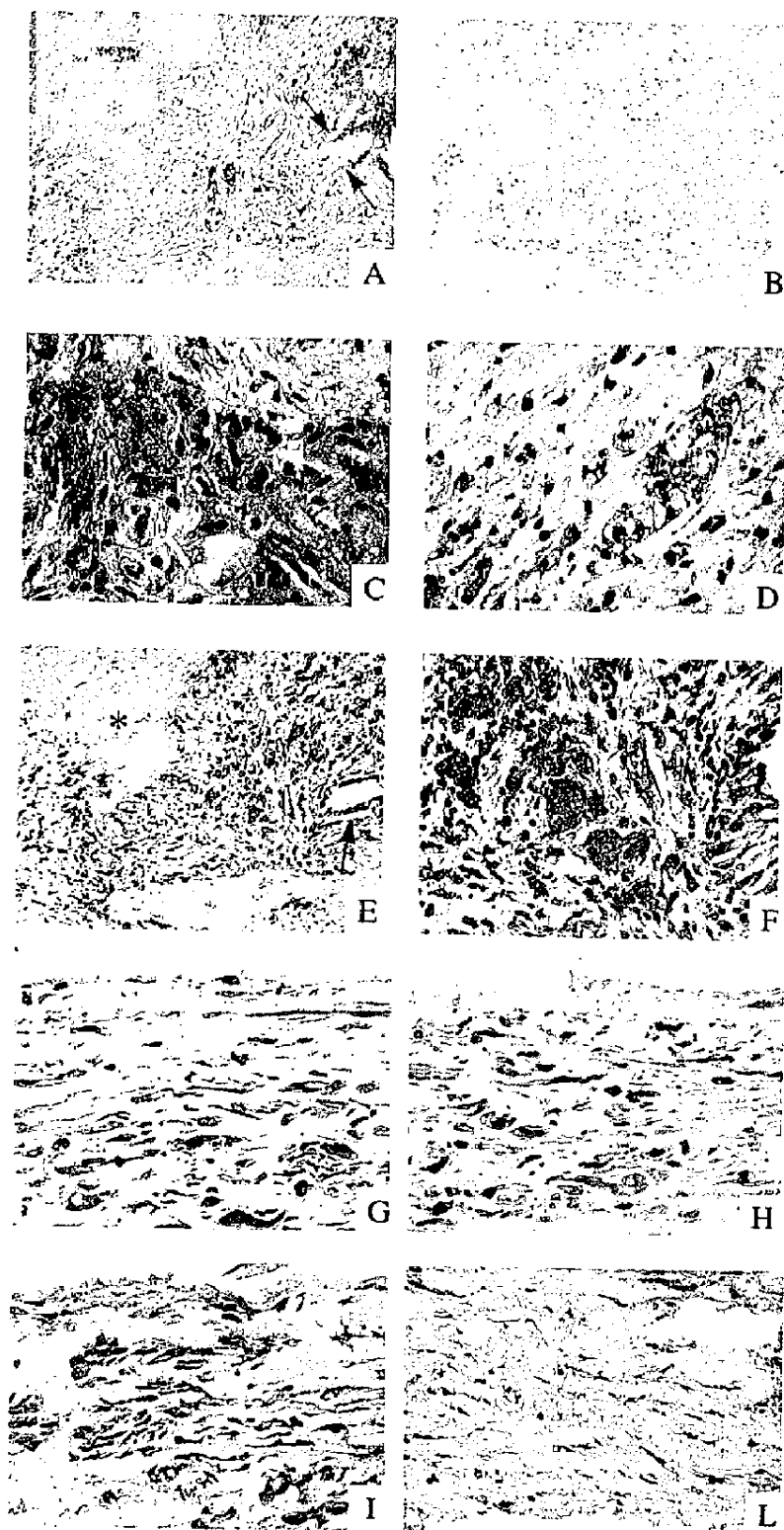
The levels of IPF<sub>2α</sub>-I in atherosclerotic plaques obtained at endarterectomy were higher than those of 8-epi PGF<sub>2α</sub> and ranged from 5.6 to 13.8 pmol/μmol phospholipid, with a median of 9.00 pmol/μmol phospholipid. The corresponding values in the normal vessels ranged from 0.16 to 0.44 pmol/μmol phospholipid with a median of 0.28 pmol/μmol phospholipid ( $P < 0.001$ ) (Fig. 2). IPF<sub>2α</sub>-I levels in the autopsy specimens ranged from 6.50 to 10.30 in the atherosclerotic segments and 0.10 to 0.72 pmol/μmol phospholipid in the corresponding normal intimal segments. A direct correlation was found between

the levels of the two isoprostanes in the atherosclerotic plaques ( $r = 0.80$ ,  $P < 0.0001$ ) obtained at atherectomy (Fig. 3). The clinical characteristics of the patients undergoing endarterectomy are represented in Table I. No correlation was observed between the levels of 8-epi PGF<sub>2α</sub> in plaque and fasting plasma concentrations of cholesterol or triglycerides (Table II). No association was evident between the increment in 8-epi PGF<sub>2α</sub> and IPF<sub>2α</sub>-I in the plaque and history of smoking habit, diabetes, or high blood pressure (Table II). 5 out of 12 patients undergoing endarterectomy were taking aspirin. No significant difference in 8-epi PGF<sub>2α</sub> levels was observed between patients taking aspirin [median(range), 2.30(1.31–3.45)], and those who did not [2.25(1.54–3.01) pmol/μmol phospholipid,  $P =$

Table II. Clinical Features of the Patients Undergoing Carotid Endarterectomy and Level of 8-epi PGF<sub>2α</sub> Found in Their Plaques

Patient	Age	Total cholesterol	Total triglycerides	High blood pressure	NIDD	Smokers	Carotid stenosis	8-epi PGF <sub>2α</sub>
	yr	mg/dl	mg/dl				%	pmol/μmol phospholipid
1	75	213	179	Yes	No	No	70%	2.950
2	70	248	200	No	Yes	No	60%	1.540
3	54	191	121	Yes	Yes	No	90%	3.450
4	71	200	69	No	No	No	80%	1.850
5	68	230	191	Yes	No	Yes	75%	2.110
6	62	264	220	Yes	No	No	95%	2.250
7*	76	210	180	No	Yes	Yes	70%	2.700
8*	70	205	190	Yes	No	Yes	80%	1.310
9	72	195	170	Yes	No	No	75%	1.850
10	60	220	135	No	No	Yes	70%	2.250
11	58	185	210	No	Yes	Yes	80%	3.010
12	64	205	200	No	Yes	No	90%	2.850

All patients were males and had suffered transient ischemic attacks. Total cholesterol and triglycerides were measured after 12 h of fasting. High blood pressure was defined as a resting, supine systolic blood pressure  $> 150$  mmHg and a diastolic blood pressure  $> 95$  mmHg. All the endarterectomy specimens had evidence consistent with fibrocalcific plaques on a carotid duplex scan. The extent of atherosclerosis was assessed from medical records, clinical symptoms, and by echo-Doppler technique. \*These patients also suffered from clinically evident peripheral vascular disease (II<sup>nd</sup> degree, La Fontaine). NIDD, non-insulin-dependent diabetes.



**Figure 4.** (A-F) Immunostaining for 8-epi  $\text{PGF}_{2\alpha}$  and cell phenotype in human carotid atherosclerotic plaque. Sections were prepared as described in Methods, and stained with the avidin-biotin-peroxidase complex. The presence of the antigen recognized by the primary antibody is indicated by a brown substrate. (A) Marked cytoplasmic positivity for anti-8-epi  $\text{PGF}_{2\alpha}$  is observed particularly in the foam cells near the lipid necrotic core (asterisk) and adjacent to cholesterol crystals (arrows) ( $\times 50$ ). (B) Negative control, with a rabbit preimmune serum, in the area of the plaque near the lipid necrotic core ( $\times 100$ ). (C) Higher power view of A that represents the area of the plaque near the cholesterol crystals in which macrophages, foam cells, and a multinucleated giant cell were positive for the anti-8-epi  $\text{PGF}_{2\alpha}$  antibody ( $\times 200$ ). (D) Foam cells strongly positive for the anti-8-epi  $\text{PGF}_{2\alpha}$  antibody ( $\times 200$ ). (E) Serial section of A near the necrotic lipid core (asterisk) and cholesterol crystals (arrow) stained with the anti-human monocyte/macrophage CD68 antibody. Some of the cells that were positive for the anti-8-epi  $\text{PGF}_{2\alpha}$  antibody are positive for CD68 antibody ( $\times 60$ ). (F) A view of E at higher magnification ( $\times 200$ ). The cells are positive for CD68 antibody ( $\times 200$ ). (G-L) Immunoreactivity for 8-epi  $\text{PGF}_{2\alpha}$  in the fibrous cap of the human atherosclerotic plaque. (G) A positive reaction for the anti-8-epi  $\text{PGF}_{2\alpha}$  antibody is detected in smooth muscle cells as well as in elongated and roundish foam cells ( $\times 200$ ). (H) A serial section of G stained with anti-CD68 antibody, specific for human monocyte/macrophage. Some of the cells positive for anti-8-epi  $\text{PGF}_{2\alpha}$  antibody are positive for anti-CD68 antibody ( $\times 200$ ). (I) A serial section of G stained with the anti- $\alpha$ -smooth muscle actin antibody. Some of the cells positive for anti-8-epi  $\text{PGF}_{2\alpha}$  antibody are positive for anti- $\alpha$ -smooth muscle actin antibody ( $\times 200$ ). (L) Immunostaining for the anti-8-epi  $\text{PGF}_{2\alpha}$  antibody is less evident in the smooth muscle cells of the tunica media ( $\times 200$ ). Sections G-I are obtained serially at slightly different levels from the same area.

0.9]. The same was true for  $\text{IPF}_{2\alpha}$ -I (data not shown). To determine if postexcision procedures artifactually produced 8-epi  $\text{PGF}_{2\alpha}$  or  $\text{IPF}_{2\alpha}$ -I from arachidonyl-containing phospholipids in the tissue, we incubated [ $^3\text{H}$ ]arachidonic acid (10  $\mu\text{g}$ ) with

the plaque at the time of the acquisition. No formation of [ $^3\text{H}$ ]8-epi  $\text{PGF}_{2\alpha}$  or [ $^3\text{H}$ ]  $\text{IPF}_{2\alpha}$ -I during processing was detected (data not shown).

**Immunohistochemistry.** Plaques examined were character-

ized by the presence of an atheromatous core of variable size with cholesterol clefts and lipid droplets. The core was covered by a fibrous cap, consisting of smooth muscle cells and interstitial tissue, often infiltrated by a variable number of macrophage-like foam cells. Such foam cells were clustered close to the lipid necrotic core, together with lymphocytes and newly formed blood vessels. Immunohistochemical studies with the anti-8-epi PGF<sub>2α</sub> antibody revealed immunoreactivity in all of the plaques analyzed. Marked cytoplasmic positivity for 8-epi PGF<sub>2α</sub> was observed in the cells near the lipid necrotic core (Fig. 4 A, *asterisk*) and in approximation to cholesterol crystals (Fig. 4 A, *arrows*). No immunostaining was detected when a preimmune rabbit serum was used as a control (Fig. 4 B). No immunoreactivity was detected when the primary antibody was omitted (data not shown). Foam cells and macrophage-like cells were strongly positive for the anti-8-epi PGF<sub>2α</sub> antibody (Fig. 4, C and D). Serial sections stained with CD68 antibody, specific for human monocytes/macrophages, demonstrated that some of the cells positive for anti-8-epi PGF<sub>2α</sub> antibody were also positive for CD68 antibody (Fig. 4, E and F). 8-epi PGF<sub>2α</sub> immunostaining was also detected in the fibrous cap of the atherosclerotic plaques (Fig. 4, G-L). A positive reaction to the anti-8-epi PGF<sub>2α</sub> antibody was detected in what appeared to be smooth muscle cells as well as in elongated and roundish foam cells (Fig. 4 G). Sections close to the fibrous cap stained with anti-CD68 or anti-α-smooth muscle actin antibodies, confirming that some of the cells positive for anti-8-epi PGF<sub>2α</sub> were either macrophages or smooth muscle cells (Fig. 4, H and I). Immunostaining for the anti-8-epi PGF<sub>2α</sub> antibody was less commonly observed in the smooth muscle cells of the tunica media (Fig. 4 L).

## Discussion

8-epi PGF<sub>2α</sub> and IPF<sub>2α</sub>-I are prostaglandin F<sub>2α</sub> isomers formed in a free radical-catalyzed manner. F<sub>2</sub>-isoprostanes are members of a larger family of eicosanoid isomers produced from arachidonic acid in this way (17, 18). 8-epi PGF<sub>2α</sub>, at least, exhibits biological activity in vitro. It is a potent vasoconstrictor, it induces platelet shape change, and is a mitogen (32-35), although it is unknown how relevant these observations are to the concentrations which pertain in vivo (36). Additional to its formation via a free radical-catalyzed pathway, 8-epi PGF<sub>2α</sub> may also be formed as a minor product of the prostaglandin G/H synthases (COXs) in vitro (25, 37, 38), although enzymatic formation is probably a trivial contributor to overall 8-epi PGF<sub>2α</sub> synthesis in vivo (21). Recently, we have developed a sensitive and specific assay for measuring another member of the F<sub>2</sub>-isoprostane family, IPF<sub>2α</sub>-I. We have demonstrated that this isoprostane is produced by humans and is excreted in urine (26). Unlike 8-epi PGF<sub>2α</sub>, this compound exhibits no COX-dependent formation in vitro, being generated solely via free radical-catalyzed peroxidation of arachidonic acid (39).

Little information is available as to oxidant injury in patients with atherosclerosis (40-42). The availability of a quantitative index of oxidant stress would afford considerable insight into the importance and treatment of this mechanism of human disease. Validation of such an approach requires several lines of evidence. Prior studies have established that F<sub>2</sub>-isoprostanes may be formed by free radical-dependent mechanisms (17, 18) and that their formation is increased in the setting of oxidant stress in vitro (43, 44). Urinary 8-epi PGF<sub>2α</sub> is a

specific representative of this class of compounds. We have observed increased excretion of 8-epi PGF<sub>2α</sub> in a variety of clinically distinct settings, which are thought to be associated with oxidant stress (21, 22, 45-47).

The present studies extend these observations by demonstrating the presence of F<sub>2</sub>-isoprostanes in a human tissue in which oxidative modification of LDL is presumed to occur, the atherosclerotic plaque. Directional atherectomy has afforded the opportunity to study constituents of atherosclerotic plaque (48). Given the expression of epitopes reflective of oxidative modification of LDL in human carotid plaques (13), we sought evidence for F<sub>2</sub>-isoprostanes. The results presented here show for the first time that two distinct isoprostanes, 8-epi PGF<sub>2α</sub> and IPF<sub>2α</sub>-I, are present in human atherosclerotic lesions. Both isoprostanes were abundant in all plaques analyzed, even though interindividual variability was apparent. By contrast, little was found in macroscopically uninvolved vascular tissue obtained from patients undergoing heart transplantation or from uninvolved segments of carotid artery obtained at autopsy. Although the transplant patients and autopsy cases were somewhat younger (43-67 yr) than those undergoing endarterectomy (54-76 yr), no age-dependent variation in 8-epi PGF<sub>2α</sub> levels or IPF<sub>2α</sub>-I was observed. Similarly, despite the theoretical contribution from COX activation to 8-epi PGF<sub>2α</sub>, no difference in the plaque content of this isoprostane was observed between patients who were taking aspirin and those who were not. These observations, together with the close correlation between plaque content of 8-epi PGF<sub>2α</sub> and IPF<sub>2α</sub>-I, strongly suggest that lipid peroxidation largely, if not completely, accounts for the 8-epi PGF<sub>2α</sub> content of plaque tissue.

The presence of 8-epi PGF<sub>2α</sub> and IPF<sub>2α</sub>-I in human plaque is consistent with the time-dependent formation of F<sub>2</sub>-isoprostanes in LDL which is oxidized in vitro (20). Indeed, we have demonstrated recently that incubation of zymosan-stimulated human monocytes with LDL in vitro results in marked, free radical-catalyzed formation of 8-epi PGF<sub>2α</sub> (25). Artifactual formation of these isoprostanes in vascular tissue ex vivo during sample preparation was excluded, since deuterated isoprostane species were not formed despite addition of [<sup>3</sup>H]arachidonic acid to the samples at initiation of the preparation procedure. Although healthy carotid tissue is unavailable to compare with the endarterectomy specimens, we performed a comparative analysis of "atherosclerotic" and "normal" regions of carotid arteries obtained at autopsy. Both F<sub>2</sub>-isoprostanes were significantly higher in the atherosclerotic segments than in uninvolved areas. The possibility of postmortem changes in the autopsy specimens cannot be excluded. However, major artifacts are unlikely, as the isoprostane levels in the "uninvolved" postmortem samples were similar to those obtained from apparently healthy vessels obtained at the time of heart transplant.

We confirmed the presence of 8-epi PGF<sub>2α</sub> by immunolocalizing this isoprostane in histological sections of endarterectomy specimens. Previous studies have demonstrated that macrophage rich-lesions from hypercholesterolemic rabbits exhibit predominantly cell-associated immunostaining with antibodies that recognize protein-bound lipid products (49, 50). Indeed, we found intense intracellular immunostaining for 8-epi PGF<sub>2α</sub> in the region of the plaque close to the lipid necrotic core. This colocalized with immunostaining for an antibody directed against the specific monocyte/macrophage epitope, CD68 (30). This region of the atherosclerotic lesion is

thought to be a locus for monocyte recruitment (50, 51) and degradation of the extracellular matrix by metalloproteinases released from these cells has been thought to contribute to plaque instability (52). 8-epi PGF<sub>2α</sub> was also detected in the tunica intima and tunica media, but not in the adventitia. Immunoreactivity was not detected in the intima associated with the endothelial cells. Rather, it was specifically localized in the sub-endothelium, associated with roundish and elongated foam cells. Immunocharacterization of these cells indicated that they were macrophages and vascular smooth muscle cells. Consistent with the likelihood that immunolocalization of 8-epi PGF<sub>2α</sub> would reflect an intracellular oxidative process, we failed to detect its formation extracellularly. Both smooth muscle cells and monocyte/macrophages may take up LDL and oxidize it (53, 54) in vitro. This process is associated with considerable generation of isoprostanes (25). These ex vivo findings suggest that similar events are likely to pertain in vivo. It is unknown if this, or other isoprostanes, may contribute to the evolution of plaque morphology, although as 8-epi PGF<sub>2α</sub> is a mitogen and a vasoconstrictor, this possibility might be investigated. Irrespective of their functional importance in atherogenesis, quantitation of isoprostanes in urine, plasma, and now in tissue may be used to guide rational dose selection of antioxidant therapy in humans.

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